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Regulatory variation in hepcidin expression as a heritable quantitative trait

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ABSTRACT

Genetic variation underlies phenotypic diversity and complex quantitative traits including heritable diseases. We hypothesized that such variation may underlie or determine intrinsic inter-individual differences in iron metabolism and may also play a role in variable phenotypes associated with iron-related diseases. Using hepcidin as a marker of iron homeostasis, we assessed sequence variation and the transcription potencies of promoter haplotypes for both hepcidin genes *mhepc1* and *mhepc2* from different strains of inbred mice. We found several single nucleotide polymorphisms (SNPs) within the promoters of both genes on one hand, and between strains on the other. With luciferase as reporter, we also found significant variation in the basal transcription of both genes. A regulatory SNP constituting an E-box in the promoter of *mhepc1* caused further expression level variation and transactivation by Upstream Stimulatory Factor, USF. Inter-strain variation in hepcidin expression correlated with established phenotypic differences in iron loading in these mice. As hepcidin is critically required for iron metabolism, we posit that variation in its expression may be a quantitative trait which determines differences in iron handling within and between mouse strains, and that this may also apply to humans. Thus, regulatory variation in hepcidin expression may be just as important as structural variation or mutations within its coding sequence.

There is increasing evidence that phenotypic differences between individuals cannot be wholly accounted for by protein structural variations, e.g., due to point mutations or SNPs within cognate genes. In most cases there are no genetic mutations at all, yet there are demonstrable phenotypic differences for example in iron metabolism. *cis*-Acting regulatory polymorphisms particularly within the non-coding regions of genes, e.g., promoters, are now considered to be just as important as protein structural variation in determining inter-individual phenotypic variation [1–3]. In other words, regulatory variation in gene transcription or differences in the levels of their protein products may underlie phenotypic variation.

Studies have shown large variations in hepatic non-heme iron levels in congenic mice [4–13], suggesting that differences in iron handling may be heritable. We reasoned that these differences may relate directly to the expression phenotypes of iron regulatory or transport proteins including the acute phase antimicrobial peptide hormone hepcidin. Hepcidin has come to prominence as the key modulator of systemic iron levels. It appears to be exquisitely sensitive to changes in systemic iron levels and inflammatory mediators [14–18], regulating iron flux by binding to and down-regulating endogenous ferroportin [19]. Because of this critical role we reasoned that differences in the levels of its expression may partly account for phenotypic variation in iron metabolism between individuals. Here using inbred mice, we found expression level polymorphisms between the two hepcidin genes on one hand, and between different mouse strains on the other. This variation correlated remarkably well with reported differences in hepatic iron levels in these mice [4–13]. In addition to *cis*-acting regulatory variation, we found that Upstream Stimulatory Factors 1 and 2 (USF1/USF2) which we previously showed to regulate hepcidin expression [20], could accentuate allelic and inter-strain differences in hepcidin transcription. We therefore posit that variation in hepcidin expression may determine phenotypic differences in iron metabolism between mice and that this may also be relevant to humans.

Materials and methods

Mouse haplotype mapping, minimal promoters and E-box constructs. Genomic DNA was isolated from liver tissue samples from different mouse strains (Harlan UK Ltd., Oxon, UK). The promoters of the two hepcidin genes were amplified by PCR and subcloned into the NheI and XhoI sites of pGL3Basic vector (Promega) as previously described [20]. All constructs were sequenced (MWG Biotech.) for authentication and comparison; *mhepc1* and *mhepc2* promoter constructs were labeled mhepc1P-luc and mhepc2P-luc, respectively.

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E-box constructs comprised duplex oligonucleotides of mhepc1P E-boxes and mhepc2P E-box, ligated into the NheI and XhoI sites of pGL3Promoter vector (Promega). The sequence for mhepc1P E-boxes was ATG CTG GGA TGC ACC CTG CAC ATG TGT ACG ACA TTG CTG GGT CC (note overlapping E-boxes), and for mhepc2P E-box, it was ATG CTG GGA TGC ACC CTG CCC ATG TGT ACG ACA TTG CTG GGT CC; a putative regulatory SNP is shaded. These E-boxes fit the consensus recognition motif CANNTG (where N is any other nucleotide), for members of the basic helix–loop–helix leucine zipper family of transcriptional regulators [21].

Haplotype/SNP interchange. Promoter constructs of C57BL/6 mice were used for site-directed mutagenesis to swap the two tandem E-boxes with the putative regulatory SNP in mhepc1P-luc (above), with the equivalent segment with one E-box from mhepc2P-luc and vice versa. Mutagenesis was performed with the QuikChange Site-Directed Mutagenesis system (Stratagene), and verified by restriction digests with AflIII (which cleaves within the E-boxes 2/3), and DNA sequencing.

Cell culture, transfection and transactivation assays. BHK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (Invitrogen). Transient transfections and reporter assays were performed as previously described [20].

Expression and purification of recombinant USF1 and USF2. PCR primers for USF1 (sense, CAT GAA TTC CAT GAA GGG GCA GCA GAA AAC AG; antisense, CAT GTC TAG A GT TGC TGT CAT TCT TGA TGA C), and USF2 (sense, CAT GAA TTC CCC CAT GGA CAT GCT GGA CCC GGG TCT G; antisense, CAT GTC TAG ACT GCC GGG TAC TCT CGC C) were used to amplify and subclone the respective cDNAs into the EcoRI–XbaI sites (underlined) of pET25b (Novagen) to generate pET25b–USF1 and pET25b–USF2, respectively. These constructs were used to transform competent BL21(DE3) pLysS cells (Novagen). Protein expression was induced by adding 1 mM IPTG to logarithmic phase cultures for 3 h. Recombinant proteins were purified on His-Spin Protein Miniprep columns (Zymo Research) or with the MagneHis system (Promega); protein purity was assessed by SDS–PAGE on a 12% gel.

Electrophoretic mobility shift assay (EMSA). Mobility shift assays were performed [20], using an E-box duplex oligonucleotide mhepcP-E-box, which is common to both genes (CTA GCG AAT TCA GAA TCA GTA CTC ACT GCC ATG TGA AAC CAG TGT GC). Oligonucleotides used for SNP interchange (see above) were similarly used. These were radiolabeled with [γ^{32}-P]ATP (Amersham Biosciences) and incubated with HepG2 cell nuclear extracts; binding specificity was determined by competition with a 100-fold molar excess of unlabeled probe, and with mutant E-box oligonucleotide (CTA GCG AAT TCA GAA TCA GTA CTC ACT GCC ATG gaA AAC CAG TGT GC; mutations are in lower case). USF binding specificity was ascertained with 4 μg antibodies to USF1 (sc-8983) or USF2 (sc-862) (Santa Cruz Biotechnology) as previously described [20]. Recombinant USF1 and USF2 were also incubated with the E-box probes encompassing the regulatory SNP described above.

Data analysis. The GraphPad Prism software (GraphPad Software Inc) was used for all data analysis.

Results and discussion

Although inbred mice provide easily accessible models for elucidating (among others) the role of hepcidin in iron homeostasis, there are intrinsic genetic differences between strains that may influence their iron handling. This is supported by several important observations including inter-strain differences in hepatic iron loads [4–13]. Since hepcidin appears to be the nexus for iron metabolism, we surmised that the levels of its expression would underlie baseline phenotypic differences in iron handling between individuals. To address this, we subcloned *mhepc1* (581 bp) and *mhepc2* (583 bp) promoters from various mouse strains as luciferase transcriptional fusions; promoter segment size selection was based on our earlier observations [20]. DNA sequence analysis revealed restriction fragment length and several single nucleotide polymorphisms between *mhepc1* and *mhepc2* promoters on one hand, and between the promoters of different mouse strains on the other (Fig. 1). Where polymorphisms were identified, they were attributed to synonymous nucleotide substitutions (A → G and C → T) or transversions (C → G and A → T). However the promoter sequences were highly conserved for most of the strains (see Supplemental Figure).

To determine differences in hepcidin expression between the mouse strains, we transfected the promoter constructs into BHK cells; luciferase assays revealed a threefold variation in promoter activity between the two genes on one hand and between mouse strains on the other (Fig. 2A and B). Basal or constitutive transcription from *mhepc2* promoters was 10-fold higher than from the corresponding *mhepc1* promoters. We also reasoned that regulatory polymorphisms could underlie heritable differences in hepcidin expression and (downstream) iron metabolism. Therefore, we searched for binding sites of transcription factors that have been shown to regulate hepcidin expression, namely USF [26], C/EBPα [22] and STAT3 [23]. Except for a SNP within the proximal STAT3 site in C3H *hepc2*, the STAT3 sites are highly conserved in all strains (see Supplementary data). A SNP (A to T transversion) within the C/EBP binding site [22] causes a disruption of this element in all *mhepc2* genes; this site overlaps the proximal STAT3 recognition sequence. We also identified a SNP within the first of two overlapping E-boxes in the *mhepc1* promoter. This SNP (hereafter referred to as SNP/−396) is a nucleotide transversion from A (in *mhepc1*) to C (in *mhepc2*) thus resulting in the disruption of E-box 2 of the five E-boxes found in the *mhepc1* promoter; consequently *mhepc2* has 4 E-boxes within the same haplotype compared with *mhepc1* (see Fig. 1). Based on our previous observations on the role of E-boxes in hepcidin regulation by USF [20], we reasoned that this polymorphism could be a regulatory SNP which may contribute to large transcriptional variations between the two genes by USF. To ascertain this, we subcloned duplex oligonucleotides of the respective sequences into a simian virus (SV40) early promoter vector, and found that they indeed had differential enhancer activity when co-transfected with USF1 or USF2 (Fig. 3A). The data showed that SNP/−396 significantly contributed to the differential USF-dependent transcription of the hepcidin genes.

To further ascertain whether SNP/−396 contributed to regulatory variation in hepcidin expression by USF within the context of the promoter, we performed a haplotype interchange using site-directed mutagenesis to replace that segment of *mhepc1* with its equivalent from *mhepc2* (both derived from C57BL/6), and vice versa; all other nucleotides in the respective promoters remained the same. This swapping resulted in a partial reversal of basal transcription or transactivation by USF (Fig. 3B), suggesting that although SNP/−396 is probably an important regulatory SNP, it may not by itself be sufficient to totally account for the differences in promoter strength or transactivation between the two genes.

To determine *trans*-acting regulatory variation between hepcidin genes from the different mouse strains, we performed co-transfection experiments with USF1/USF2. We found that both transcription factors accentuated differences in promoter strength, resulting in significant variation in hepcidin expression between *mhepc1* and *mhepc2* on one hand, and between the hepcidin genes of different mice on the other (Fig.3C and D). The primary difference in promoter activity was a 10-fold increase in *mhepc1* transactivation by USF1/USF2 compared with *mhepc2*. This reflected a total reversal of (basal) promoter activity levels observed in Fig. 2, and is probably

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                                                                  E1
mhepc1P  -521   AG.GCAGAAGGGGAATCCAACATGACAGCTACATCATATGGTCTTCACAG
mhepc2P         GCTGCAGAA.GTGACTCCAACATGAGAGCTACATCATATGGTCTTCTCGG

mhepc1P  -471   TGGTCTCCACGGTGGACTCCAGGGCTAATGCTGACACAAACTCATGGGCC
mhepc2P         TGGACTCCATGGTGGACTTCAGGGCTAAGGCTGAGACAAACTCCTGGGCC
                                                  E2/3
mhepc1P  -421   TAAA..ATGCTGGGATGCACCCTGCACATGTGTACGACATTGCTGGGTCC
mhepc2P         TAACTCATGCTGGGATGCACCCTGCCCATGTGTACGACATTGCTGGGTCC
                STAT3
mhepc1P  -371   TTCTGAGAAACCCAGTGAGTAACAGCCATACTGAAGGCACTGATAGGGGT
mhepc2P         TTCTGAGAAACCCAGTGAGTAACAGCCATACTGAGGCACTGATAGGGGT

mhepc1P  -321   AAAGGTACAGTTCTTCCACTCACCAATCCAATCACTGTTTAGGGGAAAGA
mhepc2P         AAAGGTACAGCTTTTCCACTTACCAATCCAATCACTGATTAGGGGAAAGA
                                                          E4
mhepc1P  -271   AGGGGAATTTTTCTGAGAGCCACAGTGTGACATCACAGGTGGCTGGCTGC
mhepc2P         AGGGGAATATTTCTGAGAGCCACAGGGTAGCATCACAGGTGGCTGGCTGG

mhepc1P  -221   AGGCTTGTGTCCCTGGTTCTGTCTGCCCCACCCTCTGGATGCACCTCTGC
mhepc2P         AGGCTTGTGTCCATGGCTCTGTCTGCCCCACCCTCTGGATGCACCTCTGC

mhepc1P  -171   TGGCTGTAGGTGACACAACCCTGTCCCCTGTCACTGTTCCCGCTTATCTC
mhepc2P         TGGCTGTGGGTGGCACAACCCTGTCCCCTGTCACTGTTCCCACTTATCTC
                                          STAT3
mhepc1P  -121   TCCCGCCTGTTTGGCGCCACTATTTTCTTGGAAATGAGTCAGAGCAAAAT
mhepc2P         TCCCGCCTGTTTGGCACCACTATCTTCTTGGAATTGAGTCAAGGCAAAAG
                                  E5
mhepc1P  -71    GGGGGTGGGTGAGGCGCAGGTGACCCTCCTCCCACTAGTCCCATAAAAAG
mhepc2P         GGGGGTGGAGGAGGCTCAGGTGACCGTCCTCCCACTAGTCCCATAAAAAG
                                      +1
mhepc1P  -21    GACTGGGACTGGCTCCTAGACAGCCACCACACAAGTCCTTAGACTGCACA
mhepc2P         GACTGGGACTGGCACCTAGACAGCCACCACACAAGTCCTTAGACTGCACA

mhepc1P  +30    GCAGAACAGAAGGCATGATGGCACTCAGCACTCG
mhepc2P         GCAGAACAGGAGGCATGATGGCACTCAGCACTCG
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Fig. 1. Comparison of C57BL/6J *hepc1* and *hepc2* promoters. Nucleotide number positioning is with respect to the transcription start site (+1, double-underlined; see Ref. [22]. Deletions are denoted with dots and SNPs are shaded gray. One SNP (nucleotide −396) occurs within the first of two putative E-boxes arranged in tandem in *mhepc1* promoter, resulting in A → C nucleotide transversion within the second E-box in *mhepc2*; E-boxes (labeled E1 to E5) are underlined; STAT3 sites (conserved in both genes) are over-lined. The translation initiation codon (ATG) is in bold type.

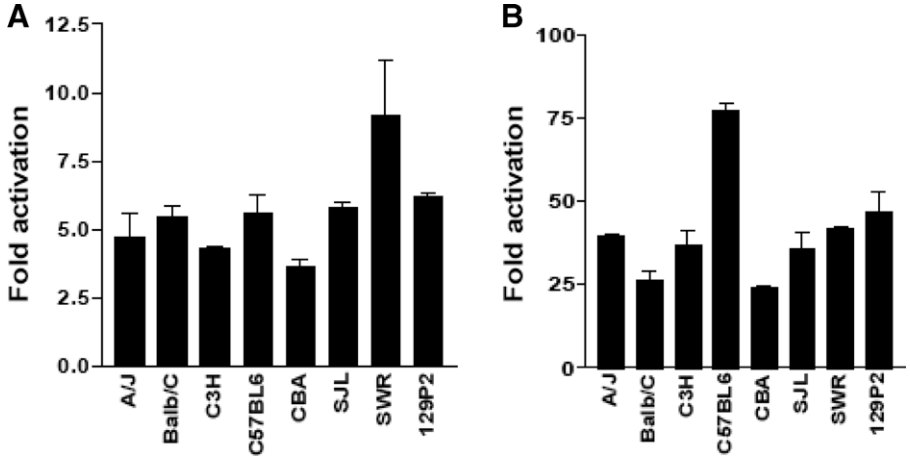

Fig. 2. Differences in hepcidin gene transcription between mouse strains. Basal promoter activities of (A) *mhepc1*, and (B) *mhepc2* promoters. Promoter constructs (100 ng) were co-transfected with 50 ng internal control vector pSVβgal, into BHK cells for 48 h. Luciferase expression was normalized with respect to β-galactosidase activity (internal control). Promoter strength was expressed as fold activation with respect to the activity of the promoter-less vector pGL3Basic, assigned an activation level of 1. Data are representative of three experiments; error bars show means ± SEM.

attributable to the regulatory SNP/−396 constituting an additional E-box (E2) in *mhepc1* which is absent in *mhepc2*. These data suggest that variations in hepcidin transcription may underlie observed differences in iron metabolism between inbred mice. It is still unclear whether *mhepc2* contributes to iron metabolism in mice but assuming that there is cross-talk or co-operativity between the

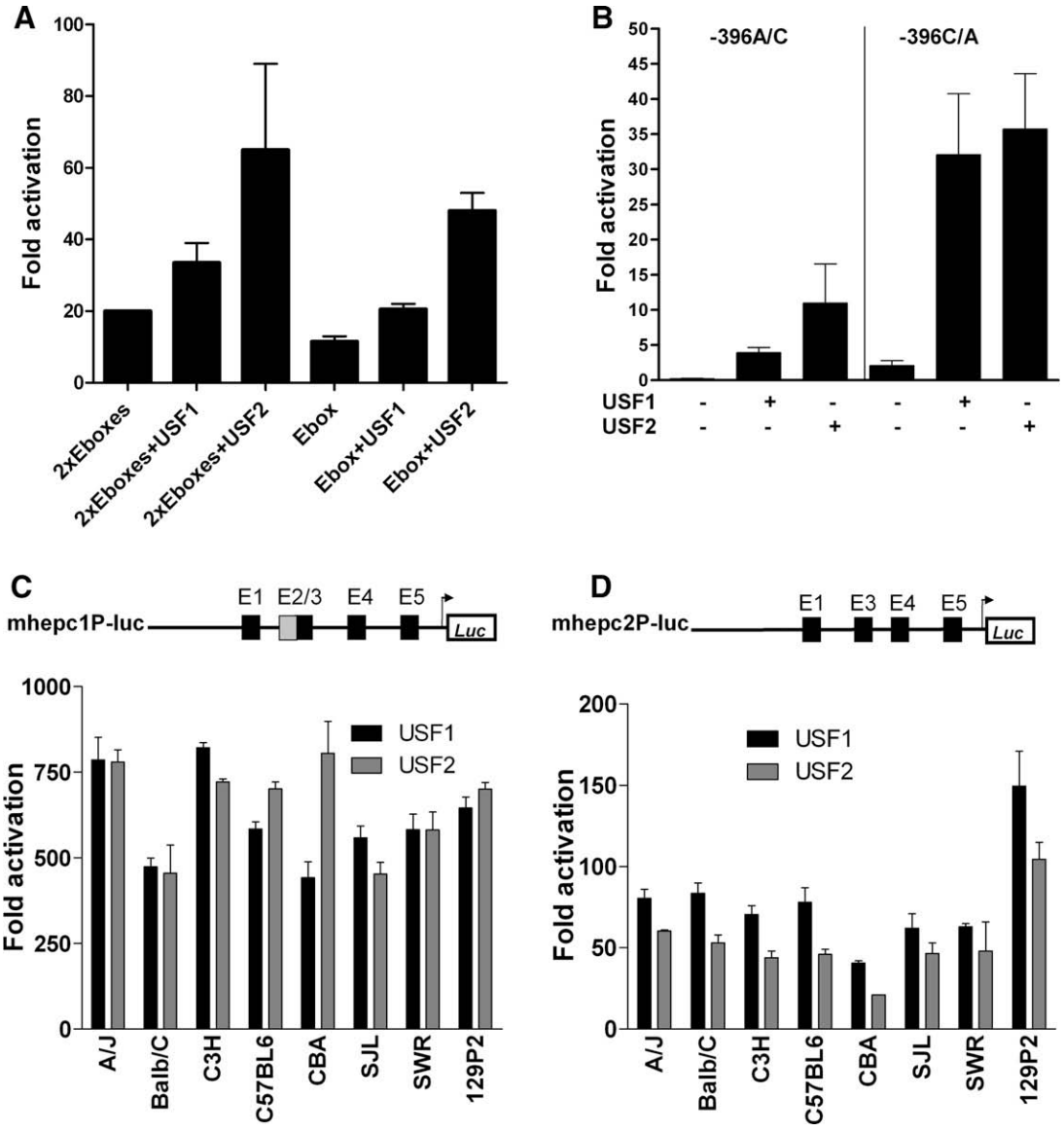

Fig. 3. *cis*- and *trans*-acting regulatory variation in hepcidin transcription. (A) Contribution of a regulatory SNP in E-box 2 in hepcidin expression variation. Duplex oligonucleotides encompassing E2/3 from *mhepc1* and E-box3 from *mhepc2* (see Fig. 1) were subcloned into the SV40 promoter vector pGL3Promoter, to generate 2xE-boxes and E-box constructs, respectively. (B) The SNP at −396 (A in E-box2 of mhepc1P-luc) was mutated to C (in mhepc2P-luc) and vice versa to give −396A/C and −396C/A, respectively; all other nucleotides remained unchanged. The constructs were co-transfected with USF1 or USF2. (C) mhepc1P-luc or (D) mhepc2P-luc constructs from various strains (note arrangement of E-boxes in the respective promoters) were transfected into BHK cells. All co-transfections employed 100 ng of each construct and 100 ng of either USF1 or USF2 plasmids, and 50 ng pSVβgal internal control. Luciferase expression was normalized to β-galactosidase and was presented as fold activation, i.e., ratios of E-box or promoter transactivation by USF1/USF2, and their respective basal activities. Data are representative of three independent experiments and are plotted as means ± SEM.

two hepcidin genes, C57BL/6 and CBA mice would, respectively, be the highest and lowest expressors based on the combined transcription of both genes in these mice. This would be entirely consistent with observations that C57BL/6 and CBA mice are at opposite ends of the iron-loading spectrum in mice [5,6,8–13]; while the former are resistant to iron-overload, CBA mice have the highest liver iron-stores [5,9]. However, it should be emphasized that due to the polygenic nature of iron metabolism, mouse liver iron stores may not necessarily directly correlate with the hepcidin expression phenotypes described here. Nonetheless, we surmise that high constitutive hepcidin expression may correlate with low hepatic non-heme iron accumulation observed in some strains and that the converse may also be true.

To determine DNA-binding propensity, we performed mobility shift assays with E-box oligonucleotides (see under Materials and methods). We found that the E-box probe with SNP/−396 in *mhepc1* bound nuclear proteins more intensely than the corresponding E-box probe in *mhepc2* (Fig. 4A). Binding specificity was confirmed by competition with excess cold probes, with mutant E-boxes and with antibodies to USF1 and USF2 (Fig. 4B). As a final proof of binding specificity, both USF1 and USF2 were expressed in bacteria, purified as hexahistidine-tagged fusion proteins and incubated in binding reactions with the E-box probes. This confirmed USF1/USF2 occupancy of the E-boxes in both hepcidin genes (Fig. 4C).

cis-Acting regulatory variation (e.g., due to nucleotide polymorphisms) contributes to heritable differences in gene expression, phenotypic diversity and the evolution of complex quantitative traits. We have shown here that this variation may also be modulated in *trans* by modifier loci, e.g., transcription factors, thus further

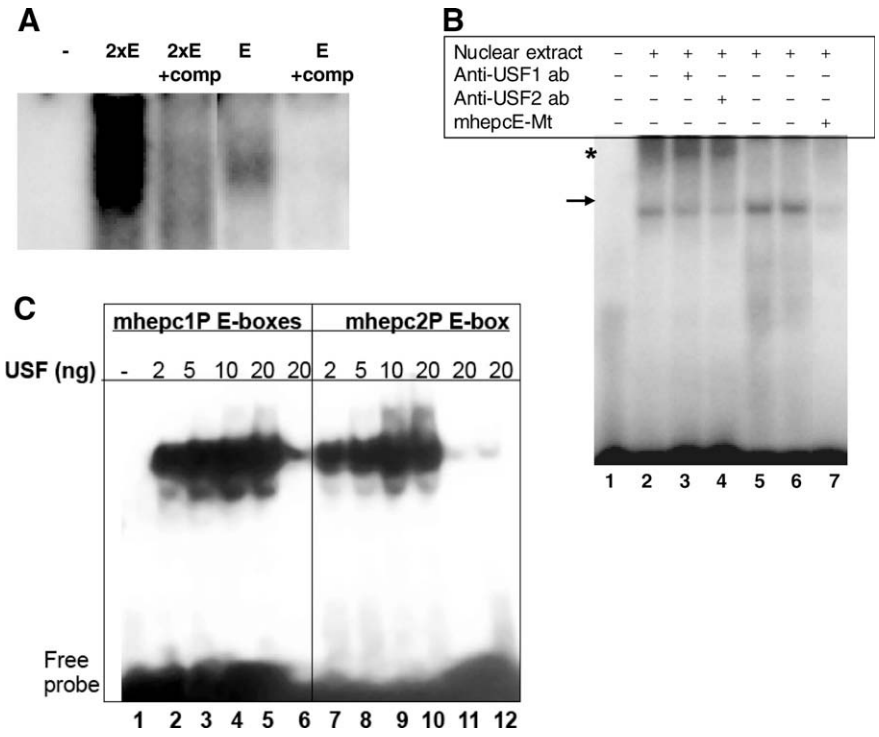

Fig. 4. EMSA. (A) HepG2 cell nuclear extracts were incubated with radiolabeled E-box probes with or without cold competitor, comp; 2xE, derived from *mhepc1* contains E-boxes E2/3; see above) while the *mhepc2* probe (E) has only E3. (B) An E-box probe common to both *mhepc1* and *mhepc2* (see above) was used for EMSA; binding specificity was assured by mutation of this E-box (mhepcE-Mt, lane7), or with USF1 and USF2 antibodies (lanes 3 and 4). Lanes 2, 5 and 6 are replicate binding reactions. (C) Increasing quantities of recombinant USF1 protein were incubated with E-box probes used in (A). In lanes 6 and 12, cold competitor was included in binding reactions; mhepcE-Mt (lane 11) and reactions without recombinant USF1, (-, lane 1), were used as controls. Similar results were obtained with recombinant USF2 (data not shown).

amplifying the effects of *cis*-acting polymorphisms. Our results suggest how such polymorphisms could determine quantitative differences in hepcidin gene expression in particular. The only known mechanism by which hepcidin controls iron metabolism is through its post-translational control of ferroportin levels. Therefore heritable variation in hepcidin expression may critically contribute to inter-individual differences in iron homeostasis [5,6,8–13]. In other words, differences in the level of hepcidin gene transcription may be just as important as structural variations or mutations within its coding sequence. This may be particularly important and pertinent to humans since only a few mutations associated with iron-overload have so far been found in the human hepcidin gene [24–27], and yet there are large inter-individual variations in iron handling and in serum hepcidin levels even between twins [28–30]. One caveat in our study worth emphasizing is that unlike mice, there is only one hepcidin gene in the human genome; therefore regulatory variation in hepcidin expression will be limited to SNPs within the cognate gene promoters rather than between hepcidin gene variants. However were such SNPs to be identified, they will provide important clues about some of the differences in iron handling between individuals. In other words, such SNPs may be informative quantitative trait loci linked to iron metabolism.

Although our observations primarily implicate regulatory variation in hepcidin expression as a possible determinant of differences in iron metabolism, there may be other levels of control, e.g., epistatic interactions between hepcidin and other independent loci including iron regulatory genes such as *HFE*. Polymorphisms at modifier loci such as the *trans*-acting factors described here may also be just as important. In other words, what our data provides is but one lever (i.e., variation in hepcidin expression) for regulation of differences in iron handling between individuals, but they do not exclusively form a basis for extrapolation of such differences. Nevertheless, our study may provide new direction for: (a) addressing the contribution of polymorphisms in iron transport genes (where they occur), and (b) determining whether there are any epistatic interactions between these genes, and how that may influence phenotypic differences in iron metabolism.

Note added in proof

While this paper was under revision, Island et al. reported a polymorphism within the bone morphogenetic protein response element in the hepcidin promoter that was associated with severe iron-overload in a human subject [31]. This supports our hypothesis that regulatory polymorphisms in this promoter may contribute to human phenotypic variation in iron metabolism and susceptibility or resistance to iron-overload.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.04.032.

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